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Progress in Identifying Epigenetic Mechanisms of Xenobiotic-Induced Non-Genotoxic Carcinogenesis

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Abstract

Determining the human relevance of structurally and functionally distinct non-genotoxic carcinogenic compounds that induce a diverse range of tissue-, gender-, strain- and species-specific tumors in animals remains a major challenge for toxicologists. Nevertheless, elucidating mechanisms of xenobiotic-induced tumors in animals can provide industry, environmental and regulatory scientists with valuable tools for cancer hazard identification and risk assessment. The discovery that aberrant epigenetic events frequently accompany genetic mutations in human cancers has stimulated efforts to deploy integrated epigenomic and transcriptomic profiling of xenobiotic-induced non-genotoxic carcinogenesis (NGC) in animal models, enabling enhanced mechanistic interpretation and novel early biomarker discovery. Recent advances in the mapping and functional characterization of mammalian tissue-specific epigenomes also provides new opportunities to characterize the cross-strain/-species chromatin architecture of non-genotoxic carcinogen effector genes and to predict their potential for modulation by xenobiotics in human tissue. Since xenobiotic-induced perturbations of gene regulation are intimately associated with the underlying DNA sequence, there is a need to integrate the impact of genotype on susceptibility to NGC. Furthermore, the potential association of xenobiotic target modulation with tumorigenic phenotypes can be assessed using genetic models and cancer genome resources. Finally, we discuss how epigenomic profiling may be used to critically assess the comparability and validity of cellular NGC models versus *in vivo*-derived tissue samples and some of key challenges associated with incorporating epigenetic mechanisms and biomarkers into cancer risk assessment.

Keywords

Non-genotoxic carcinogenesis, cancer risk assessment, epigenetics, genetics, epigenome

1. Introduction

Concerns regarding the appropriateness of extrapolating lifetime rodent carcinogenicity study findings to humans have been extensively reviewed [1-3]. If xenobiotic exposure in animals is found to be associated with either tumor induction or early indicators of neoplastic hazard, then a weight of evidence-based cancer risk assessment is generally recommended. A key contributing factor to the weight of evidence approach for xenobiotic-induced non-genotoxic carcinogenesis (NGC) is the determination of a mechanism or mode of action since this provides an entry point for subsequent assessments of potential human relevance [4,5] [Meek 2014]. A molecular basis for species-specific non-genotoxic carcinogenesis has been proposed for a number of compounds [4,5,10] [Meek 2014][Corton 2014][Cohen 2010][Cohen and Arnold 2016]. However, the diverse range of xenobiotic-induced tumor types that are typically observed in animal carcinogenicity studies, often exhibiting tissue-, gender-, strain- and/or species-specificities, make the determination of mechanism and assessment of potential relevance to humans very challenging. Furthermore, there is very little data on potential association of xenobiotic exposure with non-genotoxic carcinogenesis in humans due to the likely latency, very low incidence, and difficulty of deconvoluting environmental versus intrinsic factors for malignancy development. Some insight may be gained from somatic mutational signatures of human tumors that are associated with known mutagenic exposures [6] but there will inevitably be overlap between intrinsic and extrinsic mechanisms [7]. Despite these challenges, there is a need for rigorous cancer risk assessment of xenobiotics to which humans are exposed including treatment with novel therapeutics [8] and occupational or environmental exposure to chemicals [3].

Characterizing the molecular mechanisms underlying xenobiotic-induced non-genotoxic carcinogenesis has great potential for providing industry, environmental and regulatory scientists with valuable tools for cancer hazard identification and risk assessment. This is exemplified by phenobarbital-induced hepatocarcinogenesis where Constitutive Androstane Receptor (CAR)-mediated stimulation of mouse hepatocyte proliferation represents a mode of action that has not been reproduced in human hepatocytes *in vitro* [Hasmall and Roberts, 1999; Hirose et al., 2009; Parzefall et al., 1991]. Since there is no clear evidence for phenobarbital-associated liver cancer risk in humans (based on epidemiological data from a large number of clinical studies including long-term therapeutic treatment of epileptics; [9]), CAR-mediated liver non-genotoxic carcinogenesis is not generally considered to be human-relevant [4,10]. Humanized rodent models in which mouse livers have been engineered to express human CAR supported proliferative responses and tumor promotion following exposure to PB [11-13]. In contrast, human hepatocytes did not support hyperplastic responses to the phenobarbital in chimeric mice with humanized liver [14]. It is noteworthy that the observed plasma phenobarbital exposures in these humanized models was comparable to those obtained in human subjects receiving therapeutic doses of this drug. Thus, understanding the opposing outcomes of these models will require further characterization of: i) quantitative exposure-response relationships; ii) the influence of human nuclear receptor-mouse gene regulatory protein interactions; iii) the influence of mouse host cellular environment on grafted human hepatocytes; and iv) comparability at the molecular, biochemical and cellular levels of engineered or grafted hepatocytes to human donor-derived liver tissue. Importantly, phenobarbital induces extensive changes in chromatin modification patterns across the regulatory regions of CAR target genes in mouse

liver [15-17] and it is thus plausible that differences in the genetic and epigenetic architecture of phenobarbital effector genes play a role in determining species-specific susceptibility to CAR-mediated hepatocarcinogenesis.

Here we describe how recent advances in epigenetic regulation of the genome can be leveraged to provide new insights into molecular mechanisms of xenobiotic-induced non-genotoxic carcinogenesis, to identify early biomarkers and susceptibility factors, and to enable new approaches for assessing potential human relevance.

2. Leveraging recent advances in cancer epigenetics to enhance mechanistic interpretation and biomarkers of non-genotoxic carcinogenesis

Epigenetics describes mechanisms that operate in concert with the underlying DNA sequence to regulate gene expression and determine the overall phenotype of cell. Epigenetic marks include the methylation of DNA cytosine bases, post-translational modifications of histone proteins, nucleosome remodeling, and non-coding RNAs. These epigenetic marks are dynamically regulated by numerous enzymes and binding proteins that enable cells to read, write or erase chromatin modifications. Epigenetic formatting of the genome contributes to spatio-temporal patterns of gene expression and controls lineage choice, differentiation and cellular functions [18]. Epigenetic variation, together with genetic variation and meta-genomic variation, thus represents one of the key drivers of phenotypic variation in health and disease. The acquisition of cancer hallmarks such as sustained proliferative signaling and resistance to cell death [19] is facilitated by a combination of genome instability, mutation and epigenomic disruption [20]. Epigenetic perturbations associated with cancer etiology and progression include widespread mutations in epigenetic regulatory proteins and aberrant expression of stem cell reprogramming genes [21]. Epigenetic mechanisms of carcinogenesis that are well characterized in humans include estrogen exposure and breast cancer [22]. Importantly, from a toxicologic perspective, inflammatory responses to tissue injury and chronic exposure to environmental factors have been proposed as mechanisms for inducing cancer-predisposing epigenetic changes in vulnerable populations of somatic stem cells and progenitor compartments [23]. It is noteworthy that environmental influences on intermediary metabolism such as nutrient availability and utilisation can also result in tumor growth-promoting modifications of the epigenetic landscape by altering substrates and inhibitors of chromatin-modifying enzymes [23-27]. Epigenetic signatures of environmental exposure (e.g. to cigarette smoke) have already been identified in humans and are likely to exist for many other types of xenobiotic exposure in both humans and animals [28].

Together, these observations are stimulating efforts to investigate whether xenobiotic-induced perturbations of DNA methylation, chromatin modification, non-coding RNAs, and/or transcription factor accessibility contribute to non-genotoxic carcinogenesis [16,17,29-35].

3. Elucidating early epigenetic molecular indicators of non-genotoxic carcinogenesis

Integrated epigenomic and transcriptomic profiling of target tissues for xenobiotic-induced tumors represents a powerful approach for elucidating early molecular indicators of non-

genotoxic carcinogenesis. This is exemplified by a series of mechanistic *in vivo* rodent studies conducted within the EU IMI MARCAR consortium (www.imi-marcar.eu) in which tumor promoting doses of phenobarbital were used as a reference rodent liver tissue-specific non-genotoxic carcinogen. In addition to the anticipated activation of CAR/β-catenin signalling pathways and induction of proliferation markers, novel early biomarkers include the aberrant up-regulation of pluripotency-associated non-coding RNAs, dynamic changes in locus-specific chromatin modifications and altered transcription factor activity.

Phenobarbital progressively upregulated long non-coding RNAs encoded by an epigenetically imprinted *Dlk1-Dio3* gene cluster in mouse perivenous hepatocytes in a CAR and β-catenin dependent manner [32]. Importantly, perturbation of this gene locus has been associated with mouse stem cell pluripotency [36,37] and also with a stem-cell like phenotype in a subset of human hepatocellular carcinomas [38]. Furthermore, hepatocytes in the same perivenous region of mouse liver were recently associated with Wnt-signalling dependent stem cell-like properties [39]. Together these observations raise the intriguing possibility that PB-induces dedifferentiation/reprogramming of adult hepatocytes to a stem cell-like state during mouse hepatocarcinogenesis. The notion that functional and molecular hallmarks of pluripotent stem cells [40] might represent a valuable source of early NGC biomarkers warrants further research.

It is widely accepted that deregulation of normal DNA methylation and gene expression patterns can aid cancer cells to evolve more rapidly and thus contribute to increased invasiveness, metastatic potential and potentially drug resistance [41]. Phenobarbital induced both acute and long-lasting changes in the mouse liver DNA methylome with dynamic and reciprocal changes in 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) levels within regulatory regions of CAR and β-catenin gene targets [17]. Furthermore, early phenobarbital-induced loss of 5hmC in a specific subset of gene loci predicts subsequent aberrant promoter 5mC hypermethylation in resultant phenobarbital-promoted mouse liver tumors [34]. Recent evidence suggests that genomic loci exhibiting elevated levels of 5hmC represent dynamic chromatin states in contrast to loci marked by 5mC which are typically regarded to indicate inactive chromatin states. Consistent with this notion, the majority of enriched 5hmC is found to be associated with gene bodies, a large number of enhancer elements, and a small subset in gene promoters, in a transcription-dependent manner. Thus, the profiling of xenobiotic-induced changes in tissue-specific 5hmC levels has significant potential for classifying the mode of action of potentially carcinogenic agents [41].

Significant progress has been made in identifying target genes for a diverse range of NGC modes of action via transcriptomic profiling of rodent tissues [42,43]. Proximal regulatory transcription factors and upstream signalling pathways can often be inferred for well characterized target genes and have been extensively validated for the NGC-associated nuclear receptors CAR and Peroxisome Proliferator Activated Receptor alpha (PPARα) using biochemical, cellular and rodent genetic models. A complementary and powerful approach for identifying gene regulatory interactions underlying NGC involves computational modelling of predicted transcription factor binding sites within NGC-responsive genes and has been used to identify novel roles for E2F and ZFP161 transcription factors in regulating PB-mediated hepatocyte proliferation and subsequent tumor promotion [33].

4. Towards functional and cross-species epigenomic characterization of xenobiotic-induced non-genotoxic carcinogenesis

Importantly, multiple layers of epigenetic marks and gene regulatory factors control genome structure and function. Recent advances in the development of deep sequencing based chromatin modifications and accessibility assays enable genome-wide functional characterization of mammalian tissue-specific epigenomes. This is exemplified by the recent completion of genome-wide epigenome profiles for >100 human tissues and cell types [44,45] (http://epigenomesportal.ca/ihec/index.html) that is dramatically enhancing our understanding of genome function and regulation.

Genome-wide chromatin profiling methods including DNase I hypersensitivity and Assay for Transposase Accessible Chromatin (ATAC) assays have been integrated with histone post-translational modification mapping to identify the open cis-regulatory DNA regions (also known as the cistrome) that are recognised by trans-acting regulatory factors. Cistrome elements include gene enhancers, promoters, insulators, silencers and locus control regions. Cistrome profiling has identified hundreds of thousands of enhancer regions in the human and mouse genomes, encompassing 1-3% of the genome, and vastly outnumbering the number protein coding genes, overall highlighting the importance of the regulatory fraction of the genome [44,46,47]. Enhancers are bound by regulatory transcription factors and integrate extracellular signaling pathways with intracellular cell fate information to elicit cell type-specific transcriptional responses [48] The functional binding of regulatory transcription factors to these cis-acting accessible sites orchestrates long-range regulatory interactions in the 3D space of the nucleus, enabling cell-type specific, spatiotemporal, control of gene expression patterns which drive cell identity and function [49-51]. Furthermore, enhancers represent regulatory modules associated with the most conserved phase of vertebrate embryogenesis and are subject to conserved developmental regulation by epigenetic modifiers [52]. Importantly, the role of enhancer dysfunction in cancer has been extensively characterized [53,54]. Single enhancers and dense clusters of enhancers (known as “Super”-enhancers in the literature) appear to control the expression of core transcription factors (TFs) underlying cell identity [54-56] and are often deregulated in cancer leading to aberrant activation of growth related genes and deregulation of important lineage specific differentiation drivers [54,57].

Together these observations suggest that integrated cistrome, chromatin modification and transcriptome profiling of NGC target tissues will provide novel mechanistic insights and may provide unique opportunity to characterize the cross-strain and cross-species chromatin architecture and transcription factor accessibility of non-genotoxic carcinogen effector genes.

Although access to blood or tissue samples from humans exposed to putative NGCs is very rare, a variety of human tissue-specific epigenomes representing both healthy and disease states (http://ihec-epigenomes.org/) can in principle be leveraged as comparators to NGC target tissue epigenomes in animal models. We envisage that the potential human relevance of well-defined NGC mechanisms in animals could be explored by evaluating the degree of conservation and dynamic range of chromatin modifications/accessibility at

regulatory regions of orthologous non-genotoxic carcinogen effector genes in human tissue samples (Figure 1). It is noteworthy that comparison of mode of action-related target gene architecture in human versus animal tissue may circumvent some of the challenges associated with the comparability of cultured human cells or humanized rodent models to human tissue. Importantly, any proposed species-differences in NGC molecular signaling pathways needs to be integrated with quantitative differences in the potency and level/exposure of xenobiotics and intrinsic tissue mutation rates.

5. Influence of genotype on epigenetic mechanisms of xenobiotic-induced non-genotoxic carcinogenesis

Since xenobiotic-induced perturbations of gene regulation are intimately associated with the underlying DNA sequence, there is a need to integrate the impact of genotype on susceptibility to non-genotoxic carcinogenesis.

Distinct strains and species of preclinical animal models have been shown to widely vary in their susceptibility to xenobiotic-induced carcinogenicity. For example, mouse stocks and inbred strains can significantly differ in their susceptibility to treatment-induced liver neoplasia, with C3H males being highly sensitive compared to highly resistant C57BL/6 males, although this is likely to be based on both genetic and non-genetic factors [58]. Such strain differences in xenobiotic-induced liver tumors primarily affect tumor progression (or tumor size) and to a much lower part tumor number. Whilst specific liver tumor susceptibility genes have not yet been identified, several hepatocarcinogenesis susceptibility (Hcs) loci have been identified using mouse backcrosses and linkage analysis [59, 60]. Intriguingly, hsc3 maps within 6 megabases of the PB-responsive *Dlk1-Dio3* imprinted gene cluster on chromosome 12 [32][61]. Thus, transcriptional responses from this epigenetically imprinted gene cluster may be influenced by strain-specific genetic factors that could be further characterized via deep sequencing of appropriate inbred mouse strains.

The recent integration of human genome regulatory DNA and disease- and trait-associated genetic variants reveals a disproportionate (>80%) enrichment of disease-associated genetic variants in non-coding enhancer regions, potentially disrupting important transcription factor-based regulatory interactions in a cell-type specific manner [49,55,62,63]. These cis-acting non-coding regulatory variants range from rare to common and are associated with a broad range of phenotypic effects driven by sometimes subtle effects on target gene expression [64]. The role of non-coding sequence variants in cancer is currently being explored [65] but it seems likely that genetic variants in non-coding enhancer regions will contribute to tissue-, strain- and species-specific responses to NGC.

It is also noteworthy that most rodent carcinogenicity studies are currently performed in genetically undefined outbred stocks (e.g. CD1 mouse and/or Wistar rat) that represent a very narrow range of genetic diversity compared to humans. Whilst using more diverse panels of rodent strains might conceivably enable improved predictions of inter-individual human variability [66], the feasibility of testing xenobiotics for carcinogenicity in multiple rodent strains is limited by ethical and economic factors. Nevertheless, the genetic diversity in mice provides a powerful approach for establishing mechanism.

In the context of pharmaceutical and agrochemical product development, the ability to deploy early mechanism-based approaches for cancer hazard identification prior to entering more resource intensive and costly late phase preclinical and/or clinical studies would be highly advantageous. Molecular characterization of the potential association of drug target modulation with tumorigenic phenotypes via genetic models, germline mutation databases and cancer genome resources has recently been proposed as an innovative strategy for enhanced cancer hazard identification [8]. Where the molecular target of non-therapeutic xenobiotic NGCs can be defined through mechanistic studies, similar genetic approaches could be leveraged to help derisk the potential association of xenobiotic target modulation with tumorigenic phenotypes. For example, is there a cancer phenotype associated with genetic variants or genetic modifications of xenobiotic target genes? Are tumor suppressor-like somatic mutation spectrums associated with genes encoding xenobiotic targets in human cancers? Emerging cancer epigenome mapping resources should also be integrated to strengthen these xenobiotic target gene assessments.

6. Conclusion and Perspectives

Whilst substantial progress has been made in identifying epigenetic mechanisms and biomarkers of xenobiotic-induced non-genotoxic carcinogenesis in animal models, determining the human relevance of structurally and functionally distinct non-genotoxic carcinogenic compounds that induce a diverse range of tissue-, gender-, strain- and species-specific tumors in animals still remains a major challenge for toxicologists, although several rodent NGC modes of action are generally accepted as not being relevant for humans [4,5,10] [Meek 2014][Corton 2014][Cohen 2010][Cohen and Arnold 2016]. A number of human cellular models and humanised rodent genetic models have been deployed for NGC hazard identification but these are unlikely to fully recapitulate human tissue responses to xenobiotic exposure. Given the likely importance of epigenetic mechanisms during NGC it is particularly noteworthy that the DNA methylation profiles of mammalian cells differ from those of the primary tissues from which they were derived due to rapid reprogramming of epigenetic and transcriptional profiles following adaptation of primary cells to culture [67]. The recent refinement of cistrome, epigenome and transcriptome profiling tools for assessing cross-species conservation of tissue-specific genome functions provides a valuable opportunity to critically assess the comparability and validity of cellular NGC models versus *in vivo*-derived tissue samples. Furthermore, 5-hydroxymethylcytosine profiling is proving to be a powerful tool for cell lineage tracing [41] and thus may help deconvolute the cell type specificity of xenobiotic responses within complex tissues.

Finally, there are several challenges associated with incorporating epigenetic mechanisms and biomarkers into cancer risk assessment. These include the need to define the normal inter-individual dynamics of healthy versus disease-state epigenomes across tissues and species, and also to determine what constitutes adverse versus adaptive epigenetic changes in response to xenobiotic exposure 68,69]. However, efforts are already underway to characterize tissue-specific epigenomes from rodent stocks and strains that are regularly used for carcinogenicity testing (http://cefic lri.org/projects/c3-ed-a-comprehensive-epigenomic-profile-of-liver-tissue from-rat and mouse) and phenotypic anchoring of mechanism-based epigenetic NGC biomarkers

provides a way forward towards defining adversity at the molecular pathway level.

Figure 1. Xenobiotic signaling to chromatin and potential for species differences in regulation of NGC effector genes.

A) Potential for species differences in NGC signaling mechanisms have been extensively explored at the level of ligand-receptor interactions. For example, phenobarbital activation of the CAR nuclear receptor in hepatocytes and subsequent transcriptional up-regulation of xenobiotic response genes is highly conserved between rodents and humans. In contrast, human hepatocytes appear to be refractory to the proliferative effects of phenobarbital that are seen in rodents [Hasmall and Roberts, 1999; Hirose et al., 2009; Parzefall et al., 1991] and this is not fully accounted for by species differences in CAR-ligand interactions alone. We hypothesise that, although the molecular mechanisms responsible for the acute xenobiotic responses appear to be highly conserved across species (Target Gene a/A), secondary effectors implicated in the transduction of the extracellular signals to the nucleus and the chromatin landscape configuration of NGC effector genes are likely to contribute to strain and species differences in susceptibility to non-genotoxic carcinogens (Target Gene b/B). Such mechanistic differences in NGC molecular signaling pathways need to be integrated with potential quantitative differences in the potency/level of exposure of xenobiotics and intrinsic tissue mutation rates. B) Illustration of conserved (blue) and species-specific (red) differences in chromatin accessibility of liver tissue gene loci based on visualization of ENCODE DNase I mouse and human datasets via the UCSC browser (https://genome.ucsc.edu/; [44,70]; GEO dataset accession numbers GSE90306 and GSM1014195, John Stamatoyannopoulos, UW). Underlying DNA sequence similarity is shown as dotplots based on local genomic alignment between orthologous mouse (y axis) and human (x axis) regions (images obtained using YASS tool; [71]). Housekeeping gene (*GAPDH*), liver tissue-specific gene (*ALB*), xenobiotic response gene (*CYP2B6*; orthologous to mouse *Cyp2b10*), candidate non-coding RNA biomarker for CAR-mediated liver tumor promotion response gene (*MEG3*).

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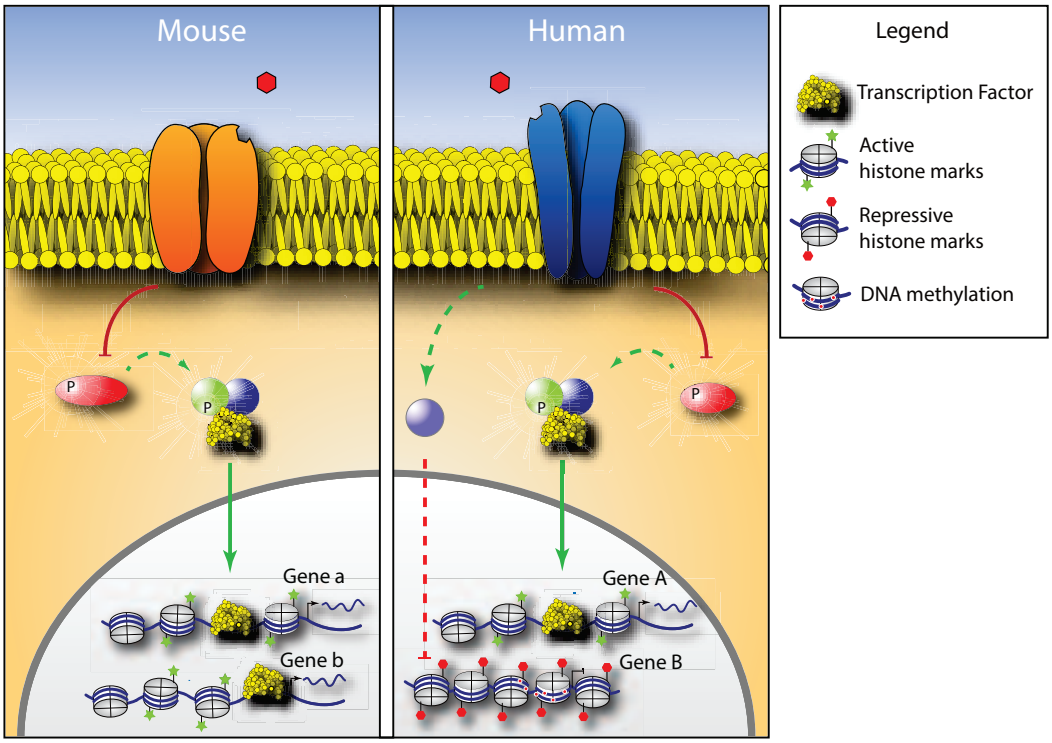
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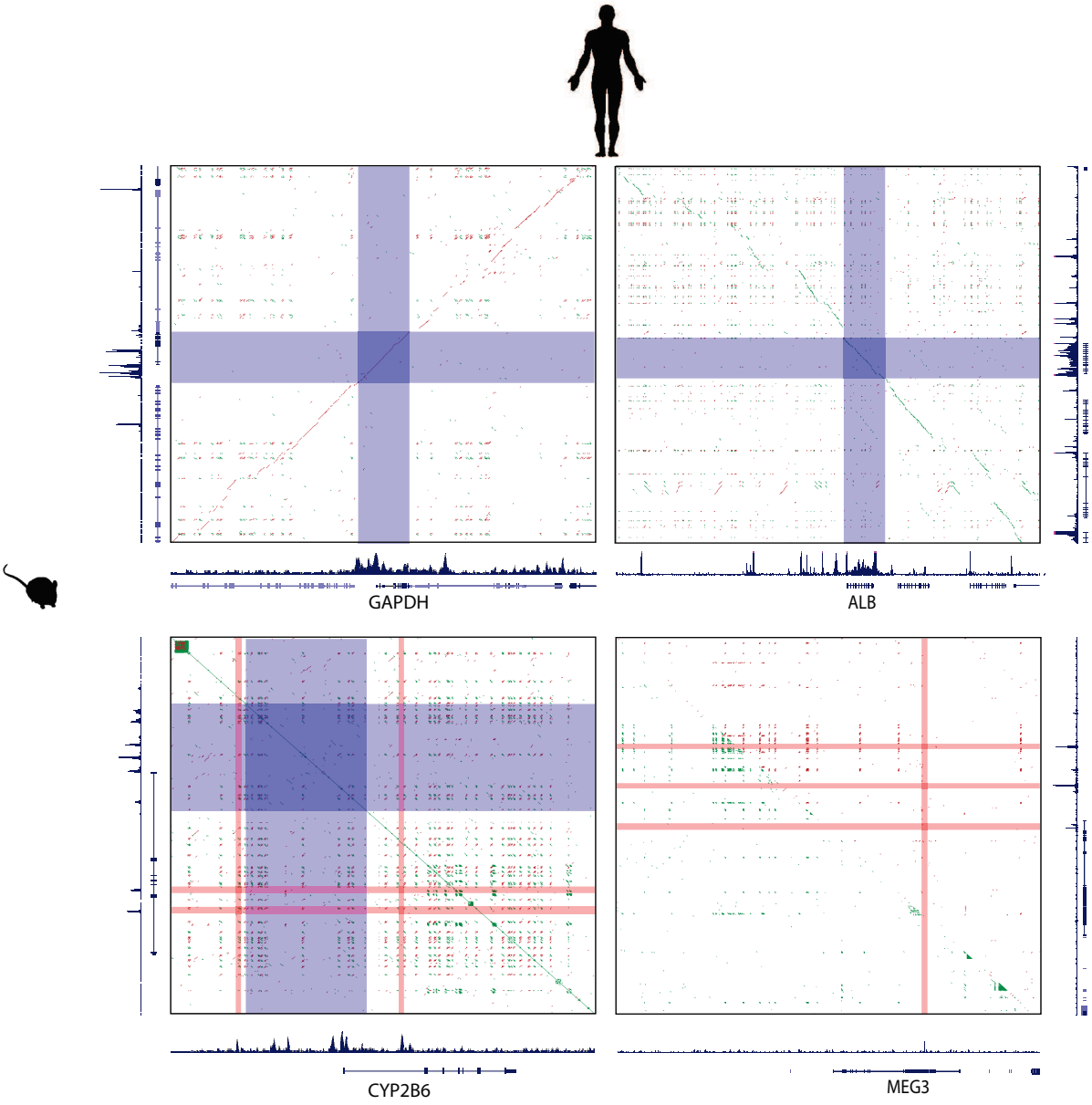
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